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TI Restoration of transcriptional activity of p53 mutants in human tumour cells by intracellular expression of anti-p53 single chain Fv fragments.
SO ONCOGENE, (1999 Jan 14) 18 (2) 551-7.
Journal code: 8711562. ISSN: 0950-9232.
AU Caron de Fromentel C; Gruel N; Venot C; Debussche L; Conseiller E; Dureuil C; Teillaud J L; Tocque B; Bracco L

TI A tumor specific single chain antibody dependent gene expression system.
SO ONCOGENE, (1999 Jan 14) 18 (2) 559-64.
Journal code: 8711562. ISSN: 0950-9232.
AU Mary M N; Venot C; Caron de Fromentel C; Debussche L; Conseiller E; Cochet O; Gruel N; Teillaud J L; Schweighoffer F; Tocque B; Bracco L

TI Mutations in p53 produce a common conformational effect that can be detected with a panel of monoclonal antibodies directed toward the central part of the p53 protein
SO **Oncogene** (1994), 9(12), 3689-94
CODEN: ONCNES; ISSN: 0950-9232

TI Antibody fragments from a 'single pot' phage display library as immunochemical reagents.
SO EMBO JOURNAL, (1994 Feb 1) 13 (3) 692-8.
Journal code: 8208664. ISSN: 0261-4189.
AU Nissim A; Hoogenboom H R; Tomlinson I M; Flynn G; Midgley C; Lane D; Winter G

TI Characterization of scFv-421, a single-chain antibody targeted to p53.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1997 Jan 13) 230 (2) 242-6.
Journal code: 0372516. ISSN: 0006-291X.
AU Jannot C B; Hynes N E

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Characterization of scFv-421, a Single-Chain Antibody Targeted to p53

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A gene encoding a single-chain antibody (scFv) which specifically binds the tumor suppressor protein p53 has been constructed from RNA of hybridoma cells producing Pab 421. scFv-421 which was expressed and purified from bacteria specifically binds p53. scFv-421, as well as the previously described scFv-FRP5 and -R1R (1), were expressed intracellularly in mammalian cells and targeted to different subcellular locations, including the nucleus, cytoplasm, and endoplasmic reticulum (ER). High levels of all ER targeted scFv proteins, but not nuclear or cytoplasmic targeted proteins, were found in transfected COS-1 cells. In an attempt to stabilize the proteins, sequences encoding the mouse immunoglobin Cκ constant domain were added to each scFv construct. This led to a moderate increase in the cytoplasmic expression of scFv-FRP5. © 1997 Academic Press

Intracellular antibodies are novel agents which can potentially be used to study or to alter the function of specific cellular proteins. We have previously targeted the receptor tyrosine kinases, EGFR and ErbB2 (1, 2), and have shown that specific ER-directed scFvs prevent the appearance of these receptors on the plasma membrane. This in turn reverts EGFR- and ErbB2-induced cellular transformation (1, 3). Most experiments have been carried out with scFvs directed to proteins of the ER since the chances of successfully altering a protein are highest in this compartment where the antibodies normally fold. However, there have been reports of scFvs expressed outside the ER (4), which prompted us to target the nuclear p53 tumor suppressor.

One of the most common genetic alterations found in human tumors is mutation of *p53* (5). Wild-type p53 has sequence-specific DNA binding activity which is lost in tumor cells with *p53* mutations. p53 DNA binding is negatively regulated by a small basic region at its C-terminus, including amino acids 363-393. Deletion of this region, or its phosphorylation by CKII or PKC activates p53 DNA binding (6, 7). The antibody Pab 421, which recognises residues 370-378 of p53, also activates p53 DNA binding (6). Interestingly, Pab 421 even has the ability to restore DNA binding activity to some p53 mutants (6, 8). These observations suggest that intracellular expression of this antibody might make it possible to alter the function of p53 directly in cell.

MATERIALS AND METHODS

Cloning and construction of scFv-421 for expression in bacteria. RNA from hybridoma cells producing Pab 421 was used to clone the heavy (VH) and light (VL) chain variable domains by standard procedures (9) with some modifications. At the 5′ and 3′ end of the VH, respectively, a SfiI cloning site and one half of the linker plus a AscI cloning site was added. At the 5′ and 3′ end of the VL, respectively, an AscI cloning site plus the other half of the linker and a NotI site were added. Purified PCR fragments were digested with SfiI/AscI for the VH and AscI/NotI for the VL, ligated together and PCR reamplified using the outstream primers VH1BACKSfiI and VKFORNotI (10). The PCR scFv fragments were gel purified, digested with SfiI/NotI, cloned into pDUCK, a modified pHEN1 vector (10) and transformed into competent *E. coli* HB2151.

Bacterial expression and purification of scFv-421. Single colonies containing the scFv insert in pDUCK were grown overnight at 37°C in 2×YT medium containing 1% Glucose and 100μg/ml Ampicillin. The cultures were diluted 100 fold in 2×YT medium containing 0.1% Glucose, and grown at 37°C until OD_{600} 0.9, then induced for 2 h at 30°C with 1mM IPTG. Bacterial extracts were prepared (11). Soluble scFv-421 was purified through a His-Zn affinity column (Pharmacia). Eluates were desalted by dialysis against PBS and concentrated with Centricon 10 (Amicon). The yield was approximately 600 μg/liter of bacterial culture.

Binding of scFv-421 to p53. Binding of purified scFv-421 to p53 was examined using two GST-p53 fusion proteins prepared by PCR amplification of WT p53 cDNA with specific primers. GST-p53N contained amino acids 1-95 of p53, GST-p53C contained amino acids 292-398 of p53. The cDNAs were amplified with p53 specific primers and BamHI and EcoRI restriction sites were included, respectively, at the 5′ and 3′ of each fragment. Amplified PCR fragments were digested with BamHI/EcoRI and cloned into a pGEX vector encoding the Glutathion-S-transferase protein. The two GST fusion proteins were prepared, purified (12), and used to coat a 96 well microtiter plate. Coating was carried out overnight at 4°C in 0.1 M carbonate buffer pH 9.6. In addition, purified bacterially expressed p53 and BSA were coated on ELISA plates. 100μl of scFv-421 (5μg/ml) was

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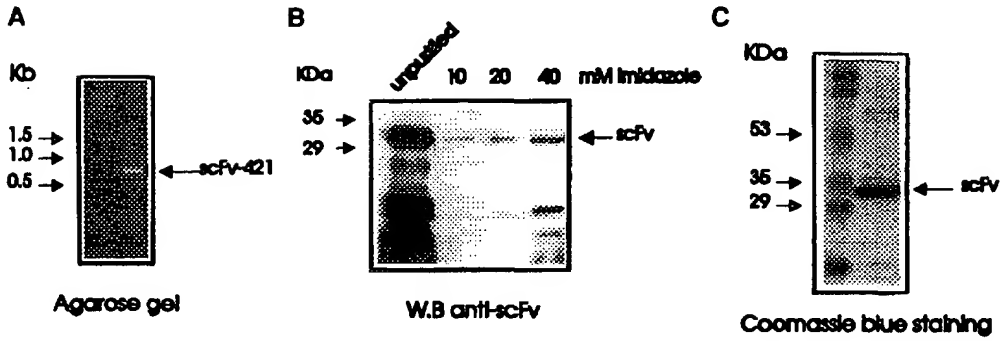

FIG. 1. (A) Molecular cloning of scFv-421. From Pab421 producing hybridoma cell RNA, VH and VL domains were amplified separately by PCR, ligated together and reamplified with outstream primers. The product was visualized on an agarose gel (1%). The arrow indicates the size of the scFv (0.7 Kb). (B) After purification of the soluble scFv through a His-Zn affinity column, an aliquot of each eluted fraction was analysed by SDS-PAGE and an immunoblot analysis was performed using a antiserum specific for scFv. (C) Approximately 3μg of affinity-purified scFv-421 was loaded on SDS-PAGE and visualized by Coomassie Blue staining.

added to each well, and the plates were incubated 1h at 4°C. Unbound scFv-421 was removed by 3 washes with PBS/0.05% Tween 20 before adding 100μl of rabbit anti-scFv serum (13). Plates were incubated 1h at room temperature, then 70μl of anti-rabbit IgG coupled to alkaline phosphatase (Sigma) was added and incubation was continued 1h at 37°C. The specifically bound scFv-421 was detected by 1h incubation at RT with a solution of 1 M Tris-HCl pH 8.0 and 0.4 mg/ml p-nitrophenyl phosphate disodium. The absorbance at 405 nm was measured.

Cloning of scFvs for expression in mammalian cells. Three different scFvs were cloned for expression in mammalian cells : scFv-421, scFv-R1 which recognises EGFR (1), and scFv-FRP5, which recognises ErbB2 (3). The scFvs cDNA were cloned into the pECE vector and were targeted to the ER using a signal peptide and the KDEL ER retention signal, as previously described (1, 3). For cytoplasmic expression, scFv cDNAs lacking the signal sequence were cloned into pECE. For some constructs, a cDNA encoding the mouse immunoglobin constant kappa (Cκ) domain was cloned at the 3' of the scFv cDNA.

Transient expression of scFv proteins in COS-1 cells. 3 μg each of pECE/scFv-421, scFv-R1 and scFv-FRP5 were transfected and transiently expressed in COS-1 cells using the lipofectamin method (Gibco BRL). After transfection the cells were returned to DMEM supplemented with 10% FCS for 60-72h before assaying for protein expression. Cell lysates were prepared in 50 mM Tris-HCl pH 7.5, 5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 10 μg/ml Aprotinin and Leupeptin, and 1 mM PMSF, and 100 μg was analyzed by Western blot with scFv specific antiserum (13).

RESULTS

The antibody Pab 421 recognizes the C-terminus of p53 (6). Hybridoma cell RNA from Pab421 producing cells was used to clone the VH and VL domain cDNAs. The two variable domains were amplified by PCR using consensus oligos and joined into one open reading frame (13). The final scFv-421 specific PCR product migrates as a 0.7 Kb fragment on an agarose gel (Fig. 1A).

In order to test the ability of scFv-421 to bind p53, the scFv was expressed in bacteria using the pDUCK expression plasmid which contains a poly Histidine tail. Soluble scFv-421 was purified from bacterial lysates by immunoaffinity chromatography using a Zn-His affinity column. The predominant protein which bound to the column and was eluted by imidazole (10, 20 and 40 mM) was the 28 kDa scFv-421 protein, as confirmed by immunoblot analysis with antiserum specific for scFv (Fig.1B). The predominant protein visualized by Coomassie blue staining was the 28 kDa scFv-421 (Fig. 1C).

Affinity purified scFv-421 was tested for its ability to bind p53 by an ELISA assay. Two GST fusion proteins, one with the N-terminal portion of p53 and one with the C-terminal portion of p53 were employed. The former should not be recognized by scFv-421 while the latter should be recognized. Intact p53 purified from bacterial extracts was also tested in the assay. The result of the ELISA is shown in Figure 2. Bacterially produced scFv-421 bound the C-terminal part of p53 (5-fold compare to control), as well as intact p53 (16-fold compare to control), but did not recognize the N-terminal portion of p53. The reason for the difference in binding observed between GST-p53C and the full p53 length is unknown, but could be due to the GST sequences in the fusion protein which might interfere with scFv-421 binding.

Intracellular expression of scFvs is potentially a rapid method to inhibit the activity of a protein. We have successfully targeted the plasma membrane receptors ErbB2 and EGFR using ER-expressed scFvs (1, 3). As a first step to test scFv-421 in eukaryotic cells it was cloned into the pECE expression vector and transiently introduced into COS cells. Since we have routinely achieved scFv expression in the ER, scFv-421 was provided with a signal peptide and a ER retention signal. To test for expression outside the ER, another version containing a strong Kozak sequence at the N-terminus in place of the signal peptide was prepared. As anticipated, high expression of scFv-421 was

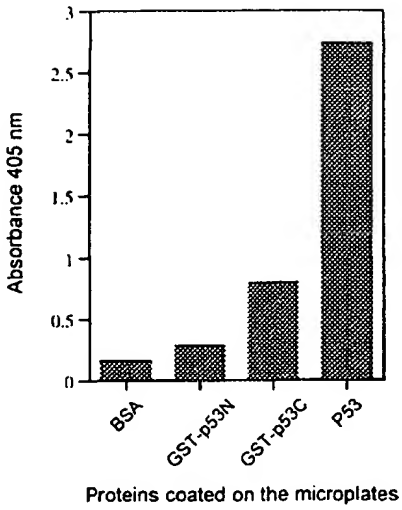

FIG. 2. Binding activity of scFv-421 tested by ELISA. Two fusion proteins containing the N-terminal part (GST-p53N), or the C-terminal part (GST-p53C) of p53, purified intact p53 and BSA were coated on microtiter plates. Purified scFv-421 was added to each well and specific binding was determined by ELISA.

achieved in the ER. However, there was no detectable expression of the cytoplasmic version (Fig. 3A left panel 421ER vs. 421cyt). We also failed to detect protein in COS cells transfected with a scFv-421 construct containing a nuclear targeting signal (not shown). High levels of the ER-directed, ErbB2 specific scFv-FRP5R were achieved (Fig. 3A right panel FRP5ER), as previously shown (2, 3). However there was no detectable scFv-FRP5 in COS cells following introduction of the cytoplasmic version of this scFv (Fig. 3A right panel FRP5cyt). The results suggest that the scFv proteins are unstable and do not accumulate in the reducing environment of the cytoplasm.

It has been reported that a kappa chain constant (Cκ) domain fused to a cytoplasmic version of Anti-Tat scFv increased the stability of the protein and made cells resistant to HIV-1 infection (4). Accordingly, sequences encoding the mouse Cκ domain were cloned at the C-terminus of the scFvs. The constructs were initially tested by coupled *in vitro* transcription and translation in rabbit reticulocyte lysates and proteins of the expected size were obtained (not shown). Expression of the different scFvs, with or without the Cκ domain, was analysed following transient transfection in COS-1 cells (Fig. 3). High levels of scFv were only seen in the cells expressing the ER-directed scFv-FRP5. Cells transfected with a vector encoding the cytoplasmic version of FRP5 yielded no protein while low levels of the cytoplasmic version containing the Cκ sequences could be detected. The level of expression of scFv-421cyt was, however, not enhanced by the Cκ sequences (not shown). There was also no detectable scFv expression in cells transfected with a cytoplasmic version of EGFR-directed scFv-R1, either with or without Cκ (Fig. 3B R1cyt vs. R1cyt-Cκ). Immunoprecipitation of lysates with a Cκ specific antiserum also failed to reveal expression of the scFv-421-Ck in transfected cells (not shown).

The sequence of the different scFvs which we have attempted to express in the cytoplasm were compared. The sequence of the scFv-421 (fig. 4) shows the hallmarks typical of other antibody variable domains (13, 14). The sequences of the framework regions and the 6 CDRs are indicated. There are various explanations for the poor expression of scFvs outside the ER. Since the proteins are foreign to the cytoplasm they may targeted for rapid degradation, e.g. via PEST sequences (15). However, putting the scFv sequences through the PEST-FIND program (available from Biomedical Informatics Unit at http://www.biu.icnet.uk/projects/pest/) revealed no PEST sequences. Each of the scFvs does have 4 Cys residues in the framework regions which, with one exception (16), are conserved in all

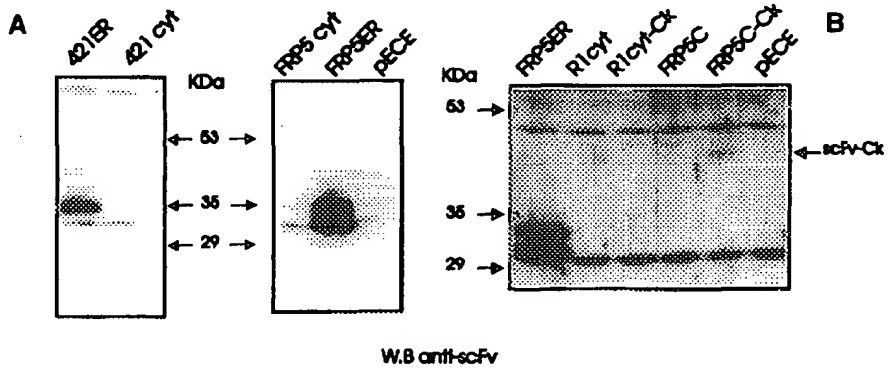

FIG. 3. Expression of different scFv constructs in COS-1 cells as determined by Western Blot analysis. (A) and (B) 100 μg of total cell extracts from COS-1 cells transiently transfected with the indicated constructs were analysed by SDS-PAGE (15%) and scFv specific expression was determined by Western analysis with specific serum. Low levels of the FRP5C-Cκ protein were detected (arrow). PECE is the control vector.

```
-------VHFR1-----*-------- VHCDR1 ---VHFR2------ -----VHCDR2-----
QESGAELVRSGASVKLSCTTSGFNIN DYYMH  WVKKRPEQGLEWIG RIDPENGDADMTRSSGV

--------VHFR3----------------*-- VHCDR3 --VHFR4---- ----linker-----
KATMTADTSSNTAYLQLSSLTSEDTAVYYCNA  GMDY  WGQGTTVTVSS GGGGSGGRASGGGGS

---- VLFR1------------* --VLCDR1-------- ---VLFR2-------   -VLCDR2-
DIELTQSPASLAVSLGQRATISC RASKSVSTSGYSYMH WNQQKPGQPPRLLIY   LVSNLES

---------VLFR3-----------------* --VLCDR3-  --VLFR4--
GVPARFSGSGSGTDFTLNIHPVEEEDAATYYC  QHIRELTRS  EGGTKLEI
```

FIG. 4. Amino-acid sequence of the p53 specific scFv-421. Variable and framework domains, and stars showing position of the 4 conserved Cys are indicated. The heavy chain of Pab421 belongs to the subgroup IB since it has 4 aa in the CDR3, the last being Tyr. Its light chain is kappa III since it has 15 aa in the CDR1 and ends with His (23).

antibodies. Thus another explanation for the lack of expression outside the ER may be due to reduction of the intrachain bridges (17).

DISCUSSION

Intracellularly expressed antibodies are novel agents which can potentially be used to study or alter many biological processes. Previous work from our laboratory has shown that ER-directed scFvs are potent inhibitors of ErbB family receptor tyrosine kinases (1, 2, 3). High expression of scFvs targeted to the ER can be routinely achieved. The potential of intracellularly expressed scFvs would, however, be greatly expanded if it were possible to consistently achieve high expression of these molecules in other compartments of the cell. The p53 protein presents an interesting target for this approach. The antibody Pab421 binds the C-terminus, altering the ability of wild-type p53 to bind DNA. Furthermore, micro-injection of Pab421 into tumor cells can restore the transcriptional activation function to a p53 mutant (18). Unfortunatly, we have been unable to achieve high levels of scFv-421 outside of the ER, making it unlikely that its nuclear or cytoplasmic expression would influence p53 activity. We investigated this phenomenon further to determine if the results were specific to scFv-421. We have shown here that two other scFvs, FRP5 and R1, which are highly expressed in the ER were not detected in the cytoplasm of transiently transfected COS cells. The addition of the Ck region to only one of the scFvs, FRP5, led to a low level of cytoplasmic expression. The results suggest that once these artificial proteins are expressed outside the oxidizing environment of the ER, they are incorrectly folded and rapidly degraded.

Disulfide bonds contribute to the structure of antibodies and the Cys residues which participate are highly conserved. In model systems it has been shown that under reducing conditions VH and VL chains are irreversibly denatured (19). Moreover, with the exception of one naturally occurring antibody which lacks a Cys in the VH domain (16), all antibodies have four conserved Cys residues, two each in the VH and VL domains (Fig. 4). Since the redox potential of the cytoplasm is higher than that of the secretory compartment (20), it is unlikely that the Cys residues are oxidized outside the ER. However, despite this reduced state, some scFvs have been expressed in the cytoplasm (17), and some expressed outside the ER did have biological effects (4) suggesting that it will be worthwhile to examine their structure more closely. Moreover, technological advances, including the production of libraries of scFvs (21) and the possibility to diversify DNA sequences *in vitro* (22) should make it feasible to screen for scFvs with a desired phenotype. Once it is possible to consistently control expression of scFvs in all cellular compartments, this approach is likely to find applications in biotechnology, gene therapy, and basic research.

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